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Identification and characterization of *Mycobacterium tuberculosis* antigens in urine of patients with active pulmonary tuberculosis: an innovative and alternative approach of antigen discovery of useful microbial molecules

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Summary

Despite the clear need to control tuberculosis, the diagnosis and prevention of this serious disease are poorly developed and have remained fundamentally unchanged for more than 50 years. Here, we introduce an innovative approach to directly identify *Mycobacterium tuberculosis* antigens produced *in vivo* in humans with tuberculosis. We combined reversed phase high performance liquid chromatography and mass spectrometry and categorize four distinct *M. tuberculosis* proteins produced presumably in lung lesions and excreted in the urine of patients with pulmonary tuberculosis. The genes (MT_1721, MT_1694, MT_2462 and MT_3444) coding for these proteins were cloned and the recombinant molecules were produced in *Escherichia coli*. The proteins were recognized by immunoglobulin G antibodies from tuberculosis patients but not from non-diseased subjects. In addition, the recombinant proteins were recognized strongly by peripheral blood mononuclear cells from healthy purified protein derivative of tuberculin-positive individuals and to a lesser extent from patients with tuberculosis. These molecules are the only proteins reported to date that are derived directly from bodily fluids of tuberculosis patients, therefore are interesting candidate antigens for the development of vaccine and/or antigen detection assay for accurate diagnosis of active tuberculosis.

Keywords: antigen detection assay, *Mycobacterium tuberculosis*, patient, purified proteins, urine

Introduction

Notwithstanding the existence of anti-*Mycobacterium tuberculosis* drugs and the widespread application of the bacille Calmette–Guérin (BCG) vaccine, global tuberculosis morbidity and mortality remain high and in many parts of the world are increasing because of co-infection with human immunodeficiency virus [1–3]. It is estimated that one-third of the world's population is infected with *M. tuberculosis* [4] and that every year 8 million new cases of tuberculosis are diagnosed and up to 2·5 million deaths are attributed to the disease [5]. BCG, the only commercially available vaccine, has been in use since the early 1920s. However, while this vaccine protects children from disseminated tuberculosis it does not prevent adult or pulmonary disease [6,7], the most common and contagious form of tuberculosis. Moreover, the already limited efficacy of BCG is highly variable in geographically distinct populations [6,8]. Effective treatment of tuberculosis requires multiple medications that must be used over extended periods of time and is complicated by multi-drug-resistant *M. tuberculosis* strains already affecting more than 50 million people around the world. Another limitation to control of tuberculosis is the lack of a sensitive and reliable diagnostic procedure. Diagnosis of active tuberculosis still relies primarily on the direct finding of the tubercle bacilli either in sputum smears or in culture, procedures that are operator-dependent and not sensitive enough to detect more than 65–70% of the disease burden.

Numerous novel vaccine and diagnostic candidates are currently being pursued. The primary approaches to their discoveries have used the immune response of patients or of resistant hosts [e.g. healthy purified protein derivative of tuberculin (PPD)-positive individuals or immunized experimental animals] as the readout of the antigen discovery strategies to select the candidate molecules [9–17]. However, an interesting alternative approach to this strategy is the direct identification of *M. tuberculosis* antigens in the bodily fluids of humans or experimental animals with active disease. Using this premise, we have reported previously the

identification of two *M. tuberculosis* antigens in the urine of infected mice and found that the recombinant versions of these antigens are potential vaccine and/or diagnostic candidates [18,19].

Here we have described the translation of this antigen discovery approach to search for *M. tuberculosis* proteins in the urine of patients with pulmonary tuberculosis. We found four unique peptides that have identical sequence homologies with the deduced amino acid sequence of four different *M. tuberculosis* proteins. The initial biological, immunological and clinical validation of these molecules are reported.

Materials and methods

Human samples

A total of 96 blood samples were evaluated in this study. These samples were collected from three distinct groups of donors. Group 1 comprised 25 patients diagnosed with pulmonary tuberculosis based on the following criteria: a clinical course consistent with active tuberculosis (e.g. fever, cough, productive sputum, suggestive chest X-ray) and culture of *M. tuberculosis* from a specimen of either sputum or pleural fluid. Nine patients were from the University Hospital, Medical School of Triângulo Mineiro (Uberaba, Minas Gerais, Brazil) and 16 patients were enrolled from Lemuel Shattuck Hospital (LSH), Jamaica Plain, MA, USA. Group 2 comprised 59 healthy PPD skin test-positive ($\geq$ 15 mm) individuals with no previous history of treatment for tuberculosis infection or disease. All subjects of this group had had recent negative chest radiographs with no evidence of active disease. These subjects were employees at the Beth Israel Deaconess Medical Center (BIDMC), Boston, MA, USA. Group 3 comprised 10 healthy PPD-negative individuals with no previous history of either BCG vaccination or known contact with tuberculosis patients. These subjects were employees at the Forsyth Institute, Boston, MA, USA. In addition to blood, urine samples were collected from the 16 tuberculosis patients at LSH and also from 16 healthy PPD^+ and PPD^- subjects. All donors were above 18 years of age and gave informed consent. The blood and urine donation protocols were approved by the Investigational Review Boards and Ethics Committees of the Medical School of Triângulo Mineiro, LSH, BIDMC and Forsyth Institute.

Mass spectroscopy

Individual human urine samples (15 ml) were loaded onto 15 ml Vivaspin 5K molecular weight cut-off filters and centrifuged at 3000 ***g*** at 4°C to reduce the retentate volume to < 2 ml. After appropriate reduction and alkylation of cysteine residues, 300 μl of urine from each patient was used for gel analysis and protein identification. These procedures were conducted at the Harvard Medical School Partners Health Care Center for Genetics, Genomics and Proteomics in Cambridge, MA, USA. Protein bands were removed and digested with trypsin. Samples were lyophilized, redissolved with 5% acetonitrile 0·1% formic acid and evaluated by mass spectrometry on a LCQ DECA XP plus Proteome X workstation (Thermofinnigan, Waltham, MA, USA). The LCQ was run in a top five configuration with one mass spectometry (MS) scan and five MS/MS scans. Dynamic exclusion was set to 1 with a limit of 30 s. Sequence analyses were performed using Sequest through the Bioworks Browser version 3·1. Sequential database searches were made using the NCBI RefSeqHuman Database using differential carbamidomethyl modified cysteines and oxidized methionines, followed by further searches using differential modifications. Secondary searches were performed using Sequest using the RefSeqHuman Gnomon predicted protein database. In this fashion, known and theoretical protein hits are found without compromising the statistical relevance of all the data. Peptide score cut-off values were chosen at Xcorr of 1·8 for singly charged ions, 2·5 for doubly charged ions and 3·0 for triply charged ions, along with deltaCN values of 0·1 and respirable suspended particles (RSP) values of 1. The cross-correlation values chosen for each peptide assure a high confidence match for the different charge states, while the deltaCN cut-off insures the uniqueness of the peptide hit. The RSP value of 1 ensured that the peptide matched the top hit in the preliminary scoring and that the peptide fragment file matched only to one protein hit.

M. tuberculosis antigens

The recombinant antigens used in these studies were produced and purified as described previously [20,21]. The yields of recombinant proteins were 10–20 mg per litre of induced bacterial culture, and purity was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. Endotoxin contamination was removed using immobilized polymyxin B (Detoxi-Gel; Pierce, Rockford, IL, USA). Endotoxin levels in purified recombinant proteins were all < 100 EU/mg protein, as indicated by the limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). PPD for *in vitro* tests was prepared as described previously [22].

Western blot

Purified recombinant proteins (100 ng) were fractionated by SDS-PAGE (4–20% gradient gel) and transferred to polyvinylidene fluoride membrane (Millipore, Medford, MA, USA). The blots were blocked overnight at 4°C with Tris-buffered saline-0·1% Tween 20 containing 1% bovine serum albumin and tested subsequently with patients and control sera as described previously [19].

Proliferation and cytokine assays

Human peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation and used in proliferation

Table 1. *Mycobacterium tuberculosis* proteins identified in the urine of patients with pulmonary tuberculosis.

Putative identification	TIGR gene annotation	Cellular role(s) (TIGR)	Gene length (bp)	MW of proteins (Da)	pI
MoaA-related protein	MT_1721	Unknown function: general	996	35 469	6·16
Ornithine carbamoyltransferase	MT_1694	Amino acid biosynthesis: glutamate family	924	33 057	5·05
Homoserine O-acetyltransferase	MT_3444	Amino acid biosynthesis: aspartate family	1140	39 798	5·39
Phosphoadenosine phosphosulphate reductase	MT_2462	Amino acid biosynthesis: serine family: central intermediary metabolism: sulphur metabolism	765	27 422	4·99

MW, molecular weight; bp, base pairs; TIGR, The Institute for Genomic Research.

([^{3}H]-thymidine incorporation) and cytokine (sandwich enzyme-linked immunosorbent assay) assays as described previously [19].

Statistical analysis

Statistical significance for responses of tuberculosis patients compared with healthy PPD$^+$ subjects was performed using Fisher's exact test. Kruskal–Wallis one-way analysis of variance followed by multiple comparisons according to Dunn's method were performed to compare the responses to individual recombinant antigens within both groups of PBMC donors. For both analyses P-values < 0·05 were considered statistically significant.

Results

Isolation of four unique *M. tuberculosis* antigens from the urine of patients with active pulmonary tuberculosis

Urine was collected from nine patients with active, culture-confirmed pulmonary tuberculosis registered at the University Hospital, Medical School of Triângulo Mineiro (Uberaba, Minas Gerais, Brazil). None of the enrolled patients had any clinical signs or symptoms or laboratory findings compatible with renal or urinary tract abnormalities. These exclusion criteria were important to rule out renal tuberculosis in these patients and therefore to support the proposed lung (but not kidney) origin of the *M. tuberculosis* antigens present in the patients' urine. None of the patients were under anti-tuberculosis therapy at the time of urine collection. Individual urine samples were analysed by mass spectrometry generating a total of approximately 400 peptide sequences. As expected, most sequences of the identified peptides had identical sequence homologies with that of human proteins. However, protein bands eluted from gel slices cut from the lower MW (~ 15–37 kDa) migration areas of the gel contained four peptide sequences that had no known homologies with human proteins but had identical sequence homologies with the deduced sequences of four different *M. tuberculosis* proteins (Table 1). Notably, all four proteins are molecules that appear to be involved in active microbial metabolism. Moreover, the gene MT_1721, in contrast to the other three genes, codes for a protein that is unique to the *M. tuberculosis* complex organisms and is apparently absent in other representative members of the *Mycobacterium* genus (Table 2).

Gene cloning and protein expression/purification of novel *M. tuberculosis* antigens and validation of their production *in vivo* during infection

The open reading frame of each of the full-length genes was amplified by polymerase chain reaction and subcloned into pET-14b expression vector. This expression vector contains a Histidine tag (His-tag) sequence before the *Nde*I cloning site,

Table 2. Distribution of the *Mycobacterium tuberculosis* genes MT_1721, MT_1694, MT_3444, and MT_2462 in representative members of the *Mycobacterium* genus*.

	Gene							
	MT_1721		MT_1694		MT_3444		MT_2462	
Organism	% Identity	ρ-value	% Identity	ρ-value	% Identity	ρ-value	% Identity	ρ-value
M. tuberculosis H37Rv	99·7	$1·5^{-176}$	88·3	$8·9^{-140}$	85·8	$1·8^{-171}$	82·3	$4·8^{-109}$
M. bovis	99·7	$1·5^{-175}$	88·3	$8·9^{-140}$	85·8	$1·8^{-171}$	82·3	$4·8^{-109}$
M. avium	31·3	$4·6^{-7}$	72	$7·1^{-115}$	68·6	$2·8^{-136}$	66·9	$5·9^{-88}$
M. smegmatis	30·6	$3·3^{-6}$	72	$4·9^{-116}$	65·9	$5·6^{-131}$	75·4	$1·0^{-81}$
M. leprae	n.r.	n.r.	75·8	$1·2^{-121}$	72·1	$1·4^{-148}$	n.r.	n.r.
Mycobacterium sp.	31	$7·1^{-8}$	71·6	$1·5^{-114}$	67·4	$4·7^{-134}$	75·9	$5·7^{-81}$

*% Identity (and respective ρ-values) of the deduced amino acid sequences coded for by the identified genes in *M. tuberculosis* CDC1551 with genes present in the indicated members of *Mycobacterium* genus were from The Institute for Genomic Research website (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). Note the unique distribution of MT_1721 (shaded areas); n.r., not reported.

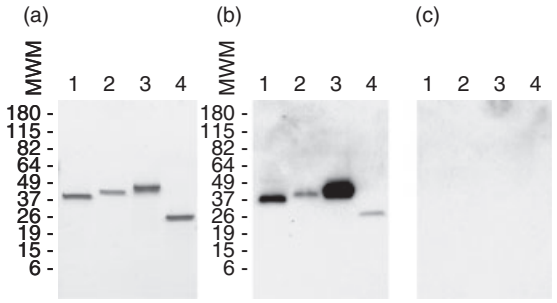

Fig. 1. Purification and characterization of the recombinant proteins coded for by MT_1721, MT_1694, MT_3444 and MT_2462. Recombinant proteins containing six Histidine tag amino terminal residues were expressed in *Escherichia coli* BL-21(DE3)/pLysS followed by purification by affinity chromatography using Ni-NTA agarose matrix. Purity was evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (4–20% gradient polyacrylamide gel) and Coomassie blue staining (a). Characterization was by Western blot analysis. Recombinant antigens (100 ng) were submitted to electrophoresis under reducing conditions in a 4–20% gradient gel and transferred to polyvinylidene fluoride membrane followed by probing with immunoglobulin G (1 μg/ml) obtained from pools of human sera from either patients with pulmonary tuberculosis (b) or normal control subjects (c). Reactivity was detected with peroxidase-labelled *Staphylococcus aureus* protein A and developed using a chemiluminescent reagent (ECL). Lane 1, rMT1721; lane 2, rMT1694; lane 3, rMT3444; lane 4, rMT2462. Numbers on the left are the molecular weights of the markers (MWM) in kDa.

thus generating a recombinant protein containing a sequence of six His residues at the N-terminus to facilitate its purification by affinity binding to an Ni-NTA agarose matrix. Recombinant protein purity was assessed by SDS-PAGE with Coomassie blue staining and is illustrated in Fig. 1a. To begin to evaluate if the discovered antigens were biologically active during disease, Western blot analyses were carried out using immunoglobulin G (IgG) antibodies purified from a pool of six sera obtained from patients with active pulmonary tuberculosis. All six patients were culture-confirmed pulmonary tuberculosis enrolled at the LSH. Figure 1b indicates that the patients' IgG clearly recognized the four antigens. No bands were seen in the blot probed with IgG antibodies purified from a pool from normal individuals (Fig. 1c). These results suggest that during disease the *M. tuberculosis* antigens MT1721, MT1694, MT3444 and MT2462 are produced in sufficient quantities to sensitize the patient's immune system to produce specific IgG antibodies, thus validating them as biologically significant molecules.

Recognition of novel *M. tuberculosis* antigens by T cells from tuberculosis patients and PPD$^+$ healthy individuals

One important requirement in vaccine development is that the interaction of a pathogen with the host boosts an immune response to antigenic components present in the vaccine. To test this requirement, PBMC were obtained from 16 patients with active tuberculosis being treated at the LSH and from 59 healthy PPD$^+$ ($\geq$ 10 mm) skin test employees of Beth Israel Deaconess Hospital. All 59 healthy PPD$^+$ individuals had multiple risk factors for having latent tuberculosis. A third group comprised healthy PPD skin test-negative individuals ($n = 10$). Recognition of the recombinant proteins was tested by antigen-induced proliferative response and production of interferon-γ. As can be seen in Fig. 2, the antigens rMT1721, rMT1694, rMT3444 and rMT2462 were recognized readily with various intensities of responses by PBMC from tuberculosis patients and healthy PPD skin test-positive individuals. However, among healthy PPD skin test-positive individuals responses to the antigens rMT1721 and rMT3444 were significantly higher ($P < 0{\cdot}05$) than the responses to the antigens rMT1694 and rMT2462. Nonetheless, these results confirm further that the four antigens are immunogenic molecules in humans sensitized with *M. tuberculosis*, hence are attractive candidates for immunological and vaccine development studies. PBMC from healthy PPD skin test-negative donors did not respond to any antigen (not shown).

Discussion

We have described recently an interesting alternative approach for the direct identification of *M. tuberculosis* antigens in bodily fluids of mice infected with this organism [18,19]. Using this approach, we identified two *M. tuberculosis* antigens in the urine of infected mice and found that the recombinant protein version of one of these antigens, when tested as a vaccine candidate, induced protection similar to that induced by BCG [19]. The second antigen proved to be of diagnostic interest [18]. These promising observations constituted the foundation of the current studies.

The translation of this strategy for antigen discovery to humans was achieved easily. Pooled urines collected from patients with well-characterized pulmonary tuberculosis yielded more than 400 peptide sequences with identical homology with human proteins and four peptide sequences with identical homology with *M. tuberculosis* proteins. Interestingly, because these four proteins are functionally active microbial molecules, they may be candidate markers of *M. tuberculosis* multiplication *in vivo*, and hence markers of active tuberculosis.

Of the four antigens, MoaA-related protein is unique in that its gene is present only in the members of the *M. tuberculosis* complex. No homologues to *M. tuberculosis* MoaA-related protein have been found in the available databases of other representative *Mycobacterium* species. Therefore, *M. tuberculosis* MoaA-related protein is a molecule that could be a useful tool for the specific diagnosis of tuberculosis because little cross-reactivity is expected to exist between this protein and other molecules present in either environmental or pathogenic non-tubercular

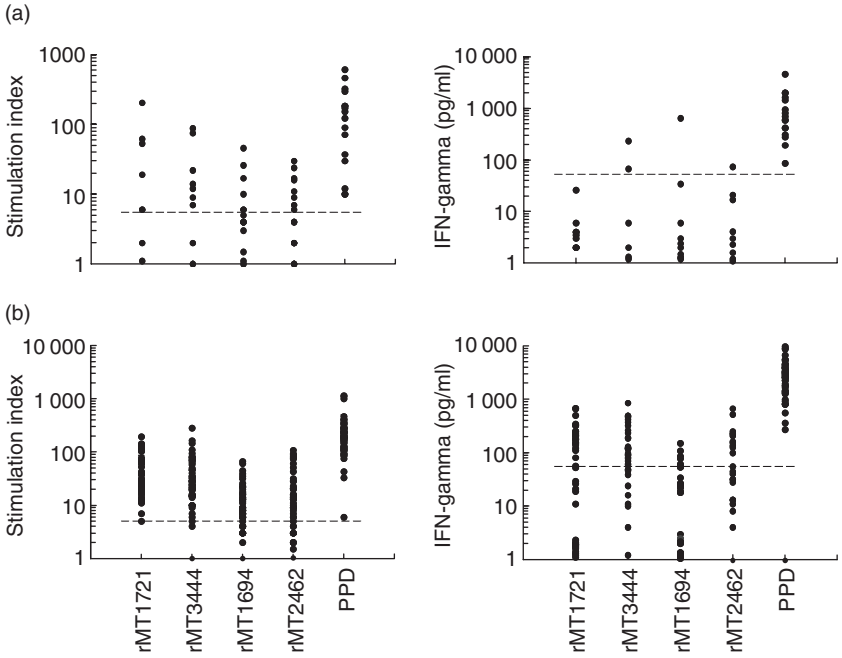

Fig. 2. Recognition of purified *Mycobacterium tuberculosis* recombinant proteins by human peripheral blood mononuclear cells (PBMC). Proliferative responses and interferon (IFN)-γ production by PBMC from (a) tuberculosis patients ($n = 16$) and (b) healthy purified protein derivative of tuberculin (PPD)$^+$ subjects ($n = 59$) were evaluated following stimulation with the indicated recombinant antigens (5 μg/ml) and PPD (2 μg/ml). Proliferation was measured by [^{3}H]-thymidine incorporation and results are expressed as stimulation index (SI). IFN-γ was measured by sandwich enzyme-linked immunosorbent assay in the culture supernatants. PBMC obtained from PPD$^-$ donors did not respond to any antigen (not shown). Dots represent individual donors. Dashed lines represent arbitrary cut-offs (SI ≥ 5; IFN-γ ≥ 50 pg/ml) which were defined using the results obtained with PBMC from PPD$^-$ donors (not shown). Pairwise multiple comparison procedures (Dunn's method) were used to compare the SI as well the levels of IFN-γ obtained for each antigen within the tuberculosis patient group and within the PPD skin test-positive healthy volunteers. Within the tuberculosis patient groups, the differences in the median values among the responses to the recombinant antigens were not great enough to exclude the possibility that differences were due to random sampling variability (SI, $P = 0{\cdot}24$; IFN-γ, $P = 0{\cdot}631$). Among the PPD skin test-positive healthy controls, significance ($P < 0{\cdot}05$) was observed for the following comparisons for SI rMT1721 × rMT1694, rMT1721 × rMT2462, rMT3444 × rMT1694 and rMT3444 × rMT2462. No significance ($P > 0{\cdot}05$) was observed between the SI for rMT1721 × rMT3444 as well as between rMT1694 × rMT2462. Significance ($P < 0{\cdot}05$) for IFN-γ production was as follows: rMT1721 × rMT1694, rMT1721 × rMT2462 and rMT3444 × rMT2462. No significance ($P > 0{\cdot}05$) was observed between rMT1721 × rMT3444, rMT1694 × rMT2462 and between rMT3444 × rMT1694. Comparisons between the responses to recombinant antigens by PBMC from tuberculosis patients *versus* healthy PPD$^+$ individuals were performed by Fisher's exact test. Significance was positive only for rMT1721 antigen in both assays (SI, $P = 0{\cdot}023$; IFN-γ production, $P = 0{\cdot}0012$).

Mycobacterium. Although the other three genes are present in all members of the *M. tuberculosis* complex they are more conserved, in that homologues (~ 70% homology at protein level) are also present in other species of *Mycobacterium*, including *M. avium* and *M. smegmatis*. However, because all four molecules are present in *M. tuberculosis* and because they are present in human bodily fluids during disease, they are of interest as potential candidates for the development of both vaccines and antigen-detection-based diagnostic assays.

Expression and purification of all four recombinant proteins were achieved with no major difficulties and their validation as genuine *M. tuberculosis* molecules produced *in vivo* during infection was performed by Western blot analysis using sera from patients with tuberculosis. This approach demonstrated clearly that patients with pulmonary tuberculosis develop specific IgG antibodies to all four *M. tuberculosis* proteins. This observation confirmed that the discovered antigens are molecules relevant to the host–pathogen interaction during disease in humans, thus supporting the hypothesis that they are interesting targets for diagnostics and/or vaccine studies.

The results obtained with human PBMC support this idea further and suggest that the antigens are potently immunogenic in humans. This suggestion is supported by the data that indicated that PBMC from PPD skin test-positive donors with high pretest probability of latent tuberculosis responded strongly to these antigens (particularly rMT1721 and rMT3444). In contrast, PBMC from 16 patients with pulmonary tuberculosis responded with much less intensity to stimulation with the recombinant antigens (particularly rMT1721, $P < 0{\cdot}05$). Although the number of patients tested

so far is relatively small to establish a solid comparison with the responses of positive PPD skin test in healthy individuals, these results are interesting in that PBMC from the latter group (presumed resistant individuals) responded strongly to the recombinant antigens.

The detection of microbial molecules in human bodily fluids of infected individuals has a strong precedent. For example, molecules from numerous viruses, bacteria such as *Streptococcus pneumoniae* or *Legionella pneumophila* and parasites such as *Entamoeba hystolitica* have long been described in various human samples (e.g. blood, mucous secretions and faeces) of patients suffering from the diseases caused by these microorganisms. Interestingly, many of these molecules were used successfully either as vaccines (e.g. for hepatitis A and B) or as tools for the development of antigen-detection-based diagnostics. Perhaps the most successful example of such tests is the commercially available test to detect *S. pyogenes* (group A) in patients with tonsillitis ('Signify Strep A', Abbot Diagnostics, Abbot Park, IL, USA). This rapid test has been used universally as a routine diagnostic of *S. pyogenes* pharyngitis for more than 10 years [23–25].

Paradoxically, although antigen detection assay has the potential to discriminate latent from active tuberculosis, the development of such a test has only recently become a matter of interest. Indeed, a test that is based on detection of *M. tuberculosis* lipoarabinomannan (LAM) in the urine of tuberculosis patients is under clinical validation. Despite some conflicting results regarding the sensitivity and specificity of this test, a significant correlation between the mycobacterial burden in sputum and LAM antigen concentrations in the patients' urines has been found [26–29]. Therefore, the proteins identified in the present studies are interesting candidates for the development of a similar antigen detection assay for the diagnosis of active tuberculosis.

In conclusion, the results presented in this work support strongly the premise of our proposed approach, i.e. the use of a powerful and reliable antigen discovery strategy to identify directly a variety of microbial vaccine and diagnostic candidate antigens in human bodily secretions. Finally, this approach should be broadly applicable to several other infectious diseases, particularly those caused by organisms that have their genome already completely sequenced.

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